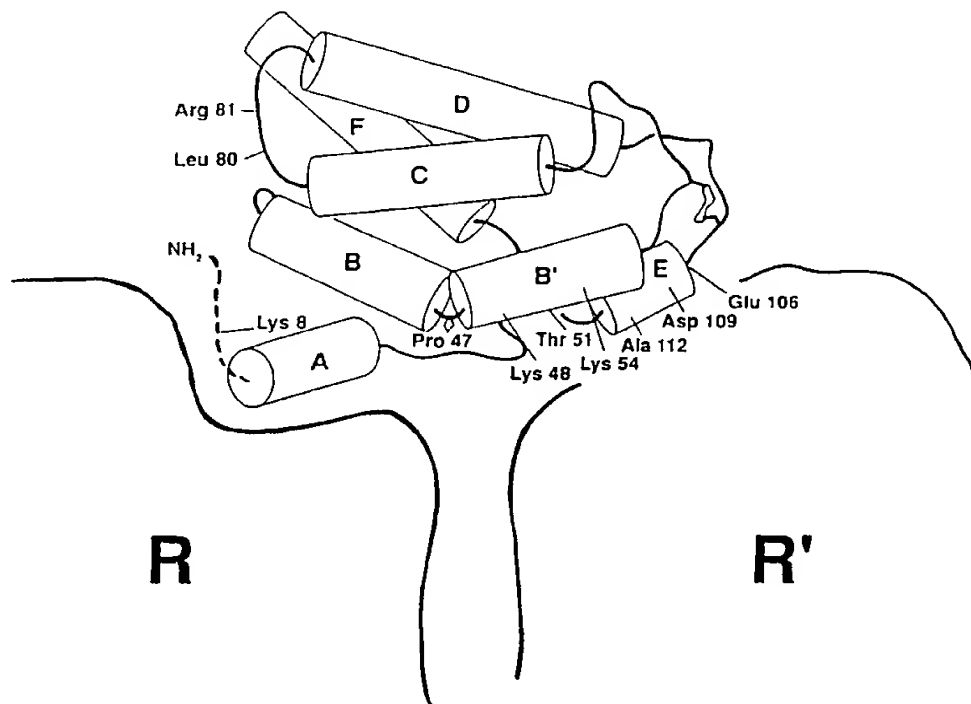




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<b>(21) International Application Number:</b> PCT/US89/02917 <b>(22) International Filing Date:</b> 5 July 1989 (05.07.89)  <b>(30) Priority data:</b> 214,998                      5 July 1988 (05.07.88)                      US  <b>(71) Applicant:</b> AMGEN INC. [US/US]; 1900 Oak Terrace Lane, Thousand Oaks, CA 91320 (US).  <b>(72) Inventors:</b> ALTROCK, Bruce, W. ; 115 Jerome Avenue, Newbury Park, CA 91320 (US). BOONE, Thomas, C. ; 3913 Elkwood, Newbury Park, CA 91320 (US). GOLDMAN, Robert, A. ; 12 Pinebrook Road, Boulder, CO 80301 (US). KENNY, William, C. ; 2654 Castillo Circle, Thousand Oaks, CA 91360 (US). STABINSKY, Yitzhak ; 82 Ahuza, 43 000 Raanana (IL).		<b>(74) Agents:</b> SCOTT, Watson, T. et al.; Cushman, Darby & Cushman, Eleventh Floor, 1615 L Street N.W., Washington, DC 20036-5601 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>

**(54) Title:** INTERLEUKIN II ANALOGS**(57) Abstract**

Interleukin II analogs and DNA sequences comprising structural genes coding for such analogs which differ from the naturally-occurring forms in terms of the identity and/or location of one or more amino acids are disclosed.

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## INTERLEUKIN II ANALOGS

The present invention relates generally to the manipulation of genetic materials and, more particularly, to the manufacture of specific DNA sequences useful in recombinant procedures to secure expression of Interleukin II analogs.

Background of the Invention

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Interleukin II ("IL-2"), a glycoprotein with a molecular weight of approximately 15,000, is a member of a group of proteins, called lymphokines, that control the body's immune response. IL-2 is produced by certain white blood cells, lectin- or antigen-activated T cells, and plays a central role in the body's immune system as a lymphocyte regulating molecule.

IL-2 has been reported to enhance thymocyte mitogenesis, to stimulate long-term in vitro growth of activated T-cell clones, to induce cytotoxic T-cell reactivity, to modulate immunological effects on activated B cells and lymphokine activated cells, to induce plaque-forming cell responses in cultures of nude mouse spleen cells, and to regulate production of gamma interferon. It also augments natural killer cell activity and mediates the recovery of the immune function of lymphocytes in selected immunodeficient states.

Additionally, in the laboratory, IL-2 is used to maintain cultures of functional monoclonal T-cells to study the molecular nature of T-cell differentiation, and to help elicit the mechanism of differentiated T-cell functions. Thus, IL-2 has application in both research and the treatment of neoplastic and immunodeficiency diseases.

IL-2 asserts its effect by binding to a specific high affinity receptor on the surface of target cells; consequently, the IL-2 molecule has become a focal point for studying receptor-effector interactions that modulate cell proliferation in the immune response.

The high affinity ( $K_D \sim 10^{-11}M$ ) receptor responsible for mediating the effect of IL-2 on target cells consists of two distinct membrane-bound proteins of size 55 kD (p55 or Tac) and 75 kD (p75); each of these two proteins can act by itself as an apparent low affinity ( $K_D \sim 10^{-8}M$ ) receptor for IL-2, and both are required for IL-2 activity. This suggests that IL-2 must bind both p55 and p75 to form a trimeric complex for activity, and by inference, that IL-2 must have two separate receptor binding sites.

The limited amount of purified native IL-2 obtainable from peripheral blood lymphocytes and tumor cell lines was an impediment to studies of the biological role of this lymphokine until the advent of recombinant production of IL-2.

Taniguchi, T., et al., Nature, 302: 305-310 (1983) described the sequence analysis, cloning, and expression of a complementary DNA coding for human IL-2, cloned from a cDNA library prepared from partially purified IL-2 mRNA from the Jurkat leukemia cell line. IL-2 was proposed to comprise 133 amino acid residues and to have a calculated molecular weight of about 15,420. Taniguchi described the cloning procedures and the expression of the cDNA for IL-2 in cultured monkey COS cells. The publication states that expression of the IL-2 cDNA in E. coli had not yet been accomplished. See also European Patent Applications 118,617, published September 19, 1984; 118,977, published September 19, 1984; and 119,621, published September 26, 1984, and U.S. Patent 4,738,927.

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Rosenberg, et al., Science, 223: 1412-1415 (1984) reported the isolation of another cDNA clone of the IL-2 gene from the Jurkat tumor cell line and from normal human peripheral blood lymphocytes. These  
5 researchers inserted the gene into E. coli, purified the polypeptide product and assayed it for biological activity. See also, Wang, et al., Science, 224, 1431-1433 (1984) referring to site-specific mutagenesis of a human IL-2 gene as well as European Patent  
10 Application 109,748, published May 30, 1984.

IL-2 modifications reported in the literature include: Ju et al., J. Biol. Chem. 262, 5723(1987); Liang et al., J. Biol. Chem. 261, 334(1986); and Miyaji et al., Agric. Biol. Chem., 51 1135(1987).

15 Considerable interest exists in the development of methods and materials for the production of large amounts of purified IL-2 analogs to replace IL-2-containing preparations currently employed in immunotherapy research.

20 It is an object of the subject invention to provide improved forms of IL-2.

It is a further object of the invention to provide IL-2 analogs having less toxicity than IL-2 preparations currently used.

25 It is a further object of the invention to provide IL-2 analogs which allow attachment of a ligand, without affecting biological activity.

It is a still further object of the invention to provide a method of purifying IL-2.

30 Other objects, features and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.

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Summary of the Invention

The subject invention relates to IL-2 analogs having modified receptor domains, and analogs having stabilized IL-2 structure. The subject invention also relates to IL-2 analogs which have been modified to permit the attachment of a ligand. More particularly the subject invention relates to a polypeptide product of the expression in a host cell of a manufactured gene, the polypeptide having an amino acid sequence represented by formula [I] below wherein at least one of the 47th, 51st, 80th, 81st, 106th, 109th, 112th, 119th, 120th, 123rd, 127th, 129th, 131st, and 133rd original amino acid residues is replaced by a substitution amino acid residue, or wherein at least two of the 8th, 47th, 48th, 51st, 54th, 80th, 81st, 106th, 109th, 112th, 119th, 120th, 121st, 123rd, 127th, 129th, 130th, 131st, 132nd, and 133rd original amino acid residues are replaced by substitution amino acid residues, and/or an additional residue is attached at the carboxy terminus, and wherein X is selected from the group consisting of Cys, Ala, and Ser:

	Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr	
	Gln Leu Gln Leu Glu His Leu Leu Leu Asp	
25	Leu Gln Met Ile Leu Asn Gly Ile Asn Asn	
	Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu	
	Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala	
	Thr Glu Leu Lys His Leu Gln Cys Leu Glu	[I]
	Glu Glu Leu Lys Pro Leu Glu Glu Val Leu	
30	Asn Leu Ala Gln Ser Lys Asn Phe His Leu	
	Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn	
	Val Ile Val Leu Glu Leu Lys Gly Ser Glu	
	Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu	
	Thr Ala Thr Ile Val Glu Phe Leu Asn Arg	
35	Trp Ile Thr Phe X Gln Ser Ile Ile Ser	
	Thr Leu Thr	

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The invention also encompasses: IL-2 analogs wherein one, two, three or more original amino acids in any helix or in any helices of IL-2 (advantageously helix A and/or helix F) have been replaced by substitution amino acids which maintain or reduce the amphiphilicity of the helix or helices; analogs wherein one, two, three or more original amino acids in helix A, B, B' and/or E have been replaced by substitution amino acids, each having a different charge from the original amino acid it replaces; and analogs wherein one, two, three or more original amino acids in helix A, B, B', C, D, E and/or F have been replaced by substitution amino acids, each having a greater preference for alpha-helical structure than the original amino acid it replaces. The invention also relates to manufactured DNA sequences encoding such polypeptides. Further, the invention relates to monoclonal antibodies specifically binding such peptides, and to methods of purifying IL-2 and IL-2 analogs.

20

#### Brief Description of the Drawings

Figure 1(a) represents the alpha carbon backbone of IL-2. Figure 1(b) is a schematic stereo drawing of IL-2; helices are represented as cylinders.

25

Figure 2 is a schematic drawing showing a possible mode of interaction of IL-2 with its receptors.

Figure 3 shows the IL-2 structure and the positions of relevant amino acids.

30

#### Detailed Description of the Invention

Novel polypeptide analogs of Interleukin II ("IL-2") have been discovered. In a first embodiment of the invention, site specific modifications of the proposed receptor binding domains of naturally occurring IL-2 are made, and alterations which stabilize IL-2

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helix structure and the overall IL-2 structure, are made. In a second embodiment, an amino acid such as an odd cysteine is incorporated into IL-2 at a location far removed from the proposed receptor binding domains but  
5 accessible to chemical reaction with other molecules.

Also provided by the present invention are manufactured genes capable of directing synthesis, in selected microbial hosts (e.g., bacteria, yeast and mammalian cells in culture), of the above noted IL-2  
10 analogs. In preferred forms of manufactured genes, the base sequence includes one or more codons selected from among alternative codons specifying the same amino acid on the basis of preferential expression characteristics for the codon in a projected host microorganism, e.g.,  
15 E. coli (see Alton et al., PCT application WO 83/04053).

Other preferred forms of manufactured genes include those wherein there is provided the nucleotide bases for a codon specifying an additional amino acid  
20 residue in the polypeptide coded for, which facilitates the direct expression in E. coli organisms (e.g., an initial Met residue). In still other preferred forms of manufactured genes, the base sequence of codons specifying the desired polypeptide is preceded by and/or  
25 followed by and/or includes one or more sequences of bases facilitating formation of expression vectors or generation of new structural genes for polypeptide analogs, i.e., sequences of bases providing for selected restriction endonuclease cleavage sites on one or both  
30 ends of the structural gene or at intermediate positions therein, and sequences providing a site for ribosome binding, e.g. CAA GGA GGT.

Also provided by the present invention are manufactured genes capable of directing the microbial  
35 expression of IL-2 analogs which differ from the naturally-occurring polypeptide in terms of the identity and/or location of one or more amino acid residues.



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In the practice of the invention, manufactured DNA sequences are inserted into viral or circular plasmid DNA vectors to form hybrid vectors and the hybrid vectors are employed to transform microbial hosts  
5 such as bacteria (e.g., E. coli), yeast cells, or mammalian cells in culture. The transformed microorganisms are thereafter grown under appropriate nutrient conditions and express the polypeptide products of the invention.

10 Also comprehended by the invention are pharmaceutical compositions comprising effective amounts of polypeptide products of the invention together with suitable diluents, adjuvants and/or carriers useful in IL-2 therapy.

15 As employed herein, the term "manufactured" as applied to a DNA sequence or gene shall designate a product chemically synthesized by assembly of nucleotide bases, synthesized by site-directed mutagenesis, or derived from the biological replication of a product  
20 thus synthesized. As such, the term is exclusive of products "synthesized" by cDNA methods or genomic cloning methodologies which involve materials which are of biological origin.

As employed herein the term "substitution  
25 amino acid" means an amino acid which replaces the naturally occurring ("original") amino acid, and which is different from the original amino acid.

In another embodiment of the invention, antibodies are provided which specifically bind the  
30 polypeptides of the subject invention but which do not cross-react with naturally occurring IL-2. These antibodies can be tagged using methods known to those skilled in the art.

The following abbreviations shall be employed  
35 herein to designate amino acids: Alanine, Ala; Arginine, Arg; Asparagine, Asn; Aspartic acid, Asp;

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Cysteine, Cys; Glutamine, Gln; Glutamic acid, Glu; Glycine, Gly; Histidine, His; Isoleucine, Ile; Leucine, Leu; Lysine, Lys; Methionine, Met; Phenylalanine, Phe; Proline, Pro; Serine, Ser; Threonine, Thr; Tryptophan, Trp; Tyrosine, Tyr; Valine, Val. The following abbreviations shall be employed for nucleotide bases: A for adenine; G for guanine; T for thymine; U for uracil; and C for cytosine.

While not wishing to be constrained to any particular theory of operation of the invention, the following detailed description is presented.

It has now been established that IL-2 is an alpha-helical protein (Fig. 1), Brandhuber et al., Science, 238, 1707 (1987) hereby incorporated by reference. It has a short helical segment near the amino terminus (residues 11 to 19; helix A in Fig. 1), followed by an extended loop; residues 33 to 56 form a helix interrupted, or "bent," near the middle by Pro 47 (hence the two segments are referenced as B and B'); following Cys 58 of the disulfide are helix C, residues 66 to 78, and D, residues 83 to 101; following Cys 105 is a short, apparently helical stretch E, residues 106 to 113, which leads into the carboxyl-terminal helix F, residues 117 to 133. There are no apparent segments of  $\beta$ -secondary structure in the molecule. The overall helical content of about 65 percent is in good agreement with estimates based on circular dichroism. The disulfide between Cys 58 and Cys 105 links two extending loops that connect the helices across the "top" (in the orientation of Fig. 1) of the molecule.

Helices B, C, D, and F form an antiparallel alpha helical bundle which differs significantly from the classical four-helix bundle represented by cytochrome c', cytochrome b<sub>562</sub>, and myohemerythrin. The packing regions of the helices are shorter, involving only three to four turns of helix, while classical four-

helix bundles usually have at least five turns in each helix. Further, the packing angles all fall in the range of 25° to 30°, and hence are somewhat larger than the average of approximately 18° found in classical  
5 four-helix bundles.

Murine IL-2 is expected to have a similar structure to recombinant human IL-2 beginning with helix A and including the proline-induced bend in helix B+B'. This is significant since recombinant human IL-2  
10 shows activity on both human and murine T cells, and recombinant murine IL-2 is reported to have a low but measurable activity on human T cells. The murine and human IL-2 sequences have 64 percent overall homology. The amino acid sequence of the mature murine protein is  
15 identical to the human sequence for the first seven residues, and then has one or more insertions, a total of 15 amino acids, relative to human, including a 12-residue poly (Gln) stretch, prior to Leu 14 of human IL-2; hence the amino terminal region of the murine  
20 protein may have significant structural differences from the human protein up to, and possibly including the first turn of, helix A. The only additional insertion in the murine sequence is between human IL-2 residues 80 and 81, in the loop connecting helices C and D.

25 The current data on IL-2 receptor binding suggest that the molecule "bridges" two receptor molecules, p55 and p75, with two independent binding sites, when bound to its high affinity receptor. Earlier work did not presage the presence of two  
30 receptor molecules; hence, modifications that affect IL-2 receptor binding do not discriminate between those involving a p55-(IL-2) interaction, a p75-(IL-2) interaction, or both.

Antibodies to peptides that cross-react with  
35 IL-2 have been used to map global regions in the IL-2 sequence likely to be important in receptor binding. In

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- particular, Kuo and Robb have presented evidence suggesting regions within the residue bounds 8 to 27 and 33 to 54 are directly involved in receptor binding, L. Kuo and R. J. Robb, J. Immunol. 137, 1538 (1986),
- 5 while Altman and colleagues found that antibodies against peptides of residues 59 to 72, 91 to 105, and 119 to 133 did not inhibit IL-2 receptor binding, A. Altman, et al., Proc. Natl. Acad. Sci. U.S.A. 81, 2176 (1984).
- 10 Ju et al. supra, have demonstrated that deletion of residues 1 to 10 of human IL-2 (the amino terminus to the beginning of helix A) reduces induction of proliferation of murine CTLL-2 cells by only 30 to 50 percent, whereas deletion of residues 1 to 20 (the amino
- 15 terminus including helix A) abolishes activity completely, Ju et al., supra. Deletion analysis of murine IL-2 shows a similar pattern of effects on proliferation activity of murine HT2 T cells, S. M. Zurawski et al., J. Immunol. 137, 3354 (1986).
- 20 Deletion of murine residues 1 to 11 or 1 to 13 (prior to helix A, assuming murine IL-2 is structurally similar to human IL-2) reduces activity by at most 50 percent. Deletions of the murine poly (Gln) section, residues 15 to 26, coupled with various changes in sequence in the
- 25 first 37 amino acids, has resulted in mutant protein with as much as one-third the specific activity of the native protein. However, deletion through murine residue 30 (corresponding to human residue 16, in the middle of helix A) reduces activity to about 0.4 percent
- 30 that of the native protein, and deletion through residue 41 (corresponding to human residue 27) abolishes activity completely.

Most of the other reported deletions that abolish activity - many of which would delete a

35 significant fraction of an internal helix in the structure or the peptide connecting them - are such that they may disrupt the overall tertiary structure of IL-2.

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Data on site-specific amino acid substitutions suffer from lack of distinction between those mutations that affect activity by destabilizing the IL-2 structure and those that directly affect receptor binding. Except  
5 for alterations that destroy the disulfide of IL-2 or modify Trp 121 (whose side chain is internal in the structure), all of the mutations shown to lower activity of human IL-2 are in sequence regions 3 to 17 and 36 to 54, Ju et al. supra, which corroborates the receptor  
10 binding regions suggested by antibody competition studies. The "down" point mutations, when placed on the IL-2 model, do not identify a specific receptor binding surface.

It is believed that Helices B, C, D, and F  
15 form a structural scaffold, and that helices A, B' and part of B, and E form the receptor binding sites of IL-2 (Fig. 2). The involvement of helix E is suggested primarily by its spatial accessibility and its proximity to regions of the molecule probably involved in receptor  
20 interactions.

IL-2 binds, through a high affinity receptor, to T-cells but will also bind and activate other immune system cells through lower affinity receptors. It is believed that activation of these other cells  
25 contributes to the observed toxic side effects of IL-2.

#### Structural Variants of Human IL-2

Alterations of the receptor binding domains of  
30 IL-2 and alterations which stabilize the IL-2 structure, produce IL-2 analogs of altered specificity towards T-cells and results in an improved IL-2 molecule possessing altered activity and/or toxicity. Included in the subject invention are IL-2 analogs wherein amino  
35 acids, advantageously hydrophilic amino acids in helices in the receptor binding domain, are replaced by amino

acids having a different charge (e.g. replacing an amino acid having a positively charged side chain by an amino acid having a negatively charged side chain, or by an amino acid having an uncharged side chain, see below).

- 5 Also encompassed by the subject invention are analogs wherein one or more amino acids which have a preference for  $\alpha$ -helical structure (see Chou and Fasman, Annu. Rev. Biochem. 47, 251(1978),

10 have been substituted into one or more of the helices of IL-2, particularly helices A, B', B, E and F, in order to stabilize the structure of the helix and of the analog as a whole. For ease in understanding the present invention, the Chou and Fasman hierarchy is presented below:

15

Preference for Forming  $\alpha$  Helix

	Glu(-)	1.51	
	Met	1.45	$H_{\alpha}$ = strong $\alpha$ former
20	Ala	1.42	
	Leu	1.21	
	Lys(+)	1.16	
	Phe	1.13	
	Gln	1.11	$h_{\alpha}$ = $\alpha$ former
25	Trp	1.08	
	Ile	1.08	
	Val	1.06	
	Asp(-)	1.01	$I_{\alpha}$ = weak $\alpha$ former
	His(+)	1.00	
30	Arg(+)	0.98	
	Thr	0.83	$i_{\alpha}$ = $\alpha$ indifferent
	Ser	0.77	
	Cys	0.70	
	Tyr	0.69	$b_{\alpha}$ = $\alpha$ breaker
35	Asn	0.67	
	Pro	0.57	$B_{\alpha}$ = strong $\alpha$ breaker
	Gly	0.57	

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The helices of IL-2 are amphiphilic helices (see Kaiser et al., PNAS, 80, 1137-1143(1983) and Kaiser et al., Science, 223, 249-255(1984).

This amphiphilic helical structure is shared by several cytotoxic peptides such as mellitin, pardoxin, and maganins. More specifically, the F helix is very amphiphilic and some of the amino acids in the F helix do not have a strong preference for the  $\alpha$ -helical structure, and it is believed that the interaction of the hydrophobic face of the F helix with the C and D helices provides the energy required to maintain the F helix sequence in its helical form. IL-2 analogs have been constructed containing altered helix sequences in which the amino acid replacements were selected to contain residues with a greater preference for a  $\alpha$ -helical structure (e.g. Asn  $\rightarrow$  Gln, Trp  $\rightarrow$  Phe, Ser  $\rightarrow$  Gln) and consequently the helices do not require strong amphiphilic interactions to maintain their helical structure, and thus the amphiphilicity of the helix can be maintained (for example an amino acid having a hydrophilic side chain being changed to a different amino acid having a hydrophilic side chain - the three substitutions noted above maintain amphiphilicity) or altered (for example by replacement of an amino acid having a hydrophilic side chain with one having a hydrophobic side chain), by amino acid substitution.

Analogues have been constructed to which other molecules can be covalently attached without damaging activity. These analogues are used to attach toxins, reporter groups, or antiviral or other therapeutic compounds which lead to the development of IL-2 conjugates of therapeutic importance as well as the production of specifically labeled IL-2 for the development of sensitive biological assays. The most convenient way to achieve these site specific conjugations is through the introduction of an odd

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cysteine residue which will present a unique reactive sulphhydryl group. Since the naturally occurring odd cysteine (Cys 125), is buried and most likely unreactive, the strategy focuses on the incorporation of a chemically accessible cysteine into (Ala<sup>125</sup>)IL-2.

The subject invention includes alterations where an additional amino acid is inserted between existing amino acids as an alternative to replacement of an existing amino acid.

10

I. Alterations in the Receptor Binding Domain of IL-2 and Alterations which Stabilized the IL-2 Structure

Hydrophilic amino acids in alpha helical regions (designated A, B', B and E in Figures 1, 2 and 3), implicated in high affinity receptor interactions, are among the candidate locations for amino acid changes. Substitution of other amino acids (particularly amino acids which alter the charge on the helix surface which interacts with the receptor) at these sites alter the spectrum of activities of the molecule by affecting receptor binding properties. Such changes also alter the ability of the molecule to interact with receptors on different cell types. Those cells are then more or less susceptible to IL-2 depending on the character of their surface IL-2 receptors. For example, cells bearing only the p75 component of the IL-2 receptor do not respond to an IL-2 species wherein the p75 binding domain has been altered such that the molecule can no longer bind to the receptor in a biologically meaningful way. The introduction of such selectivity to the IL-2 cell stimulation process allows for a greater therapeutic index for the material (e.g. by a reduction in undesirable side effects which results from stimulation of one cell type while at the same time retaining the ability of IL-2 to stimulate an appropriate effector cell



type which, in turn, limits disease). For example, certain alterations in helices in the receptor binding domain produce IL-2 analogs having a reduced capacity to induce induction of lymphokines such as INF- $\gamma$ , IL-1 and TNF, but having equivalent biological activity, relative to (Ala<sup>125</sup>)IL-2 or natural IL-2.

For ease in understanding the present invention, the side chains of the following amino acids are generally considered to be nonpolar (hydrophobic):  
10 Ile, Leu, Met, Phe, Pro, Trp, Tyr, and Val;  
the side chains of the following amino acids are polar (hydrophilic) but uncharged:

Ala, Asn, Cys, Gln, Gly, Ser, and Thr;  
the side chains of the following amino acids are  
15 hydrophilic and positively charged:

Arg, His, and Lys;  
and the side chains of the following amino acids are hydrophilic and negatively charged:

Asp and Glu.

20 See also Hopp and Woods, PNAS 78 No. 6, 3824-3828(1981);  
Kyte and Doolittle, J. Molec. Biol., 157, 105-132(1982);  
and Parker et al., Biochemistry, 25 5425-5432(1986).

## 25 A. Alterations of the E Helix

The short E helix is involved in receptor binding. Therefore, the E helix is an excellent target for mutations that alter receptor binding. The side  
30 groups of three amino acids, Glu 106, Asp 109, and Ala 112, protrude out from the E helix for possible interaction with the receptor. Figure 3 shows the IL-2 structure, the proposed receptor binding domains, and positions of the relevant amino acids. Receptor -  
35 effector (e.g. IL-2) interactions are in part mediated by electrostatic interactions between charged groups on

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the receptor with oppositely charged groups on the effector. The E helix has no positively charged amino acid side chains and three negatively charged side chains (Glu 106, Asp 109 and Glu 110). Alterations of the type of charge in the E helix of IL-2 thus alters binding efficiency. An Asp 109 → Lys 109 substitution alters receptor binding with a single mutation. Asp 109 protrudes from the center of the E helix and is a conserved amino acid. A Glu 106, Asp 109, Ala 112 → Lys 106, Lys 109, Lys 112 substitution radically changes the E helix from a negatively charged to positively charged surface. Changing either Glu 106 or Asp 109 (acidic residues) to Lys results in a net change in charge of +2 of the E helix. Changing a neutral residue (Ala 112) to Lys gives a change of +1. Changing all three residues to Lys results in a change in charge of +5. These changes can be made individually or together. Further, substitutions can be made with amino acids which have a greater preference for the α-helical structure.

#### B. Alterations of the B and B' Helices

Three amino acids, Lys 48, Thr 51, and Lys 54 protrude out from the B' helix for possible interaction with receptor. The B' helix has one negatively charged amino acid side chain (Glu 52), and four positively charged side chains (Lys 48, Lys 49, Lys 54, and His 55). A Lys 48, Thr 51, Lys 54 → Glu 48, Asp 51, Glu 54 substitution radically changes the B' helix from a positively charged to negatively charged surface. Similar changes in charge can be made in the B helix. As in the case with the E helix alterations, these changes can be done individually or together. As with the other helices of IL-2, substitutions can be made with amino acids which have a greater preference for the α-helical structure.

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C. Pro 47 → Gly 47

The breakage of the B helix into the B and B' helices is a unique structural feature of IL-2. It is believed that Pro 47 rigidly holds B and B' helices at the optimum angle to allow the B' residues to interact properly with the receptor. A Pro 47 → Gly 47 substitution gives more flexibility to the hinge joint separating the B and B' helices which changes the positioning of the important B' residues and ultimately alters the IL-2-receptor interaction. Substitutions by other amino acids at position 47 are also encompassed by the subject invention.

15 D. Alterations of the A Helix

The A helix is involved in receptor binding. The A helix has one negatively charged amino acid side chain (Glu 115), and one positively charged side chain (His 16). As with the the other helices of IL-2, amino acids which have a preference for the α-helical structure can be substituted to strengthen the structure of the A helix. Alteration of charge in the A helix as in the E, B, and B' helices, is also encompassed by the subject invention. Lastly, amino acid changes which maintain or reduce the amphiphilicity of the A helix are also included in the subject invention.

E. Alteration of the F Helix

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The F helix is an amphiphilic helix. Some of the amino acids in the F helix do not have a strong preference for the α-helical structure and it is believed that the interaction of the hydrophobic face of the F helix with the C and D helices provides the energy required to maintain the F helix in its helical form.

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IL-2 analogs have been constructed containing altered F helix sequences in which the amino acid replacements were selected to contain residues with a higher preference for an  $\alpha$ -helical structure (e.g. Asn  $\rightarrow$  Gln, Trp  $\rightarrow$  Phe, Ser  $\rightarrow$  Gln) and consequently the F helix does not require strong amphiphilic interactions to maintain its helical structure, and thus the amphiphilicity of the helix can be maintained or reduced.

10 II. Alterations in IL-2 Which Allow Attachment of a Ligand

The sites of substitutions which allow attachment of a ligand include the carboxyl terminus as well as portions of the molecule with surface exposure chosen such as to minimally perturb the structure of active IL-2. For example, addition, insertion or substitution of a cysteine residue provides a sulphydryl group which can be chemically conjugated to:

20 a) radiolabeled moieties (for assay, imaging). Conjugation of reporter groups to IL-2 allow for the rapid and sensitive detection of the resulting active IL-2 analogs. These conjugations are used as the key component in the development of sensitive IL-2 biological assays;

b) enzymatic moieties (for assay, directed therapeutic delivery);

30 c) toxins (for selective cell killing, in vitro or in vivo). Conjugation of IL-2 to cytotoxic agents should direct the toxins to cells which present IL-2 receptors. These conjugations are useful in treating certain leukemias, transplant rejections, autoimmune or altered immune states, or other cell populations of pathological significance;

35 d) drugs (directed therapeutic delivery, e.g. AZT for AIDS). Cells that are susceptible to HIV infection are, for the most part, the cells that carry

- 19 -

IL-2 receptors. Conjugation of IL-2 to retrovirus inhibitors, such as AZT (a reverse transcriptase inhibitor) direct the inhibitor to the infected cells and provide a mechanism for the internalization of the inhibitor through the IL-2 receptor. These conjugations are useful in treating AIDS and other related diseases;

5 e) antibodies or mitogens (selective cell targeting, e.g. helper T cells using OKT 4 or equivalent, and/or selective cell activation to an IL-2 responsive state); or the like.

Examination of the x-ray structure of IL-2 reveals that it is possible to conjugate other molecules to IL-2 either at the carboxy terminus or in the region spanning amino acids 79 to 82, without interfering with the receptor binding domain of the IL-2 molecule (see Figure 3). A free cysteine residue has been incorporated into (Ala<sup>125</sup>)IL-2 (an IL-2 analog containing one disulfide bond but no free cysteines) at the carboxy terminus and/or the 79 to 82 region of IL-2. Incorporation of the free cysteine residue(s) at these positions, accomplished by modifying the recombinant IL-2 gene, allows for the specific chemical conjugation of other molecules to IL-2, by reaction with the free sulphydryl group(s), in a manner that does not affect the binding of IL-2 to its cell surface receptors.

#### A. Alterations in the Carboxy Terminus Region

The carboxy terminus region of IL-2 is not involved in receptor binding and is a good location for the incorporation of an odd cysteine. Leu 132 was chosen because it is close to the C-terminus (next to last amino acid) and it is an unconserved residue when comparing human, bovine, and murine IL-2 sequences. An analog in which cysteine is simply added to the carboxy end of IL-2 ((Cys<sup>134</sup>)IL-2) also accomplishes the goal of incorporating an odd cysteine at the C-terminus.

- 20 -

### B. Alterations in the Region Between C and D Helices

The alignment of human, bovine, and murine IL-2 sequences show that, relative to the human sequence, the bovine and murine sequences contain an insertion between amino acids 80 and 81. The amino acids are part of a four amino acid loop (amino acids 79 to 82) that connects the C and D helices of IL-2. This observation coupled with the fact that this region of the molecule is far removed from the proposed receptor binding domains and other Cys residues in the molecule, makes this an ideal location for an insertion of an odd cysteine residue. Substitutions such as Leu 80 +Cys 80 are also encompassed by the invention.

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### C. Alterations in the Amino-Terminal Region

A Lys 8 +Cys 8 substitution at a nonconserved residue provides a reactive group near the amino terminus.

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### IV. Active or Competitive IL-2 Fragments

Some structural component or combination of components of IL-2 have retained or lost biological properties of intact IL-2 together with the ability to bind IL-2 receptor(s). Such a peptide is useful in place of intact IL-2 or as an antagonist of its action(s). Peptides for this application include at least one of the A, B, B' and E helices, for example, the invention includes A and E helical regions which preserve their own internal symmetry as species isolated and apart from the conformational constraints of the intact parent molecule. Such isolated structures bind to components of the IL-2 receptor and either do or do not have IL-2 biological activity. Such structures retain activity on

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only a subset of IL-2 responsive cells allowing for greater precision in manipulating the IL-2 response. Such isolated structures have lost biological activity but function as competitive inhibitors of IL-2 binding  
5 and are useful in antagonizing physiological states involving a stimulant effect of IL-2. Such structures function to up or down regulate IL-2 receptors and, thereby influence cellular receptivity to IL-2.

## 10 V. Additional Alterations

Additional analogs which are encompassed by the present invention include the analogs of IL-2 noted above further characterized by the presence of one or  
15 more of the following alterations in the amino acid sequence of naturally-occurring IL-2.

- (a) deletion and/or replacement of amino acid residues providing sites of intramolecular folding;
- (b) deletion of terminal amino acid residues;
- 20 (c) addition of amino acid residues to terminal amino acid residues;
- (d) deletion and/or replacement of amino acid residues providing sites of hydrolytic instability under highly acidic conditions;
- 25 (e) replacement of amino acid residues with glutamine residues;
- (f) replacement of amino acid residues with phenylalanine residues;
- (g) deletion and/or replacement of tryptophan  
30 residues;
- (h) deletion and/or replacement of asparagine residues;
- (i) deletion and/or replacement of cysteine residues;
- 35 (j) replacement of amino acid residues with serine residues; and

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(k) replacement of amino acid residues with alanine residues.

#### IL-2 Purification and Removal of Pyrogens

5 IL-2 and IL-2 analogs are very hydrophobic proteins and as such have a propensity to bind pyrogens which are also hydrophobic. These peptides can be formulated in a stable, monomeric form at acidic pH  
10 values. Under these conditions, pyrogens tend to form higher molecular weight aggregates, even though the monomeric molecular weight of pyrogens is comparable to that of IL-2. Thus, manufacturing procedures which fractionate proteins on the basis of size are used to  
15 separate monomeric IL-2 from aggregated forms of pyrogens. A suitable procedure for carrying out this step is by ultrafiltration through YM-30 (Amicon) membranes. Repeated dilution and ultrafiltration can be used to enhance the yield of IL-2. Glucose, mannitol,  
20 or another bulking agent, can be added as a toxicity modifier and the desired concentration of the IL-2 can be obtained by concentration by ultrafiltration or by dilution with an appropriate buffer. Pyrogens can also be separated from monomeric IL-2 by size exclusion  
25 chromatography, e.g. using Sephadex G-75. Detergents (e.g. laurate, sarcosine, sodium dodecyl sulfate) render this method ineffective since, in the presence of detergents, pyrogens are reduced to lower molecular weight forms and have apparent molecular weights  
30 comparable to IL-2. Procedures which might be expected to remove pyrogens from IL-2 solutions, such as ion exchange chromatography and hydrophobic chromatography proved to be ineffective under the conditions examined. The method of the subject invention is easy  
35 to scale up and is very cost effective.



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The following examples illustrate practice of the invention in the manufacture of the DNA sequences coding for microbial expression of IL-2 and polypeptide analogs thereof. Also illustrated is the construction of expression vectors for microbial expression of desired polypeptides.

## EXAMPLE 1

## Construction of Oligonucleotide Sequences

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This example is directed to the procedure employed in the synthesis of oligonucleotide sequences employed to manufacture the IL-2 analog genes according to the invention.

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Oligonucleotide sequences were synthesized using a four-step procedure with several intermediate washes. Syntheses were performed on Applied Biosystems (ABI) Model 380 automated synthesizers using ABI supplied reagents. Polymer bound dimethoxyltrityl protected nucleoside in support columns was first stripped of its 5'-protecting group (dimethoxyltrityl) using 3% trichloroacetic acid in dichloromethane for one minute. The polymer was then washed with acetonitrile. The washed polymer was then rinsed with dry acetonitrile, placed under argon and then treated in the condensation step using tetrazole in acetonitrile with the protected nucleoside phosphoramidite in acetonitrile. This reaction was allowed to proceed for 2.0 minutes. The reactants were then removed by filtration. This was followed by capping the unreacted 5'-hydroxyl groups using a solution prepared by mixing one part of a mixture containing acetic anhydride, 2,6-lutidine and tetrahydrofuran (1:1:8), and one part 6.5% dimethylaminopyridine in tetrahydrofuran. After one minute the capping solution was removed and the polymer was treated for 1.5 minutes with an oxidizing solution (0.1 M  $I_2$  in  $H_2O$ /2,6-lutidine/THF, 1:10:40).

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This was followed by an acetonitrile rinse. The cycle began again with a trichloroacetic acid/methylene chloride deprotection and was repeated until the desired oligonucleotide sequence was obtained.

5           The final oligonucleotide chain was treated with fresh concentrated ammonia at room temperature for 2.0 hours. After decanting the solution from the polymer, the concentrated ammonia solution was heated at 60°C for 16 hours in a sealed tube.

10           Each oligonucleotide solution was extracted with 1-butanol and ethyl ether and the concentration of each solution was determined with a spectrophotometer (260nm). 5.0 OD units of each oligonucleotide were dried down for preparative electrophoresis and loaded into a  
15 15% polyacrylamide, 7 molar urea gel. After electrophoresis, the product band was visualized by UV shadowing, cut from the gel, extracted and then desalted on a G-50 Sephadex column to yield the purified oligonucleotide.

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## EXAMPLE 2

Construction of IL-2 Analogs  
by Oligonucleotide Site-Directed Mutagenesis

This example relates to the use of recombinant  
25 methods to generate analogs of IL-2. Site directed mutagenesis procedures according to Souza, et al., published PCT Application No. WO 85/00817, published February 28, 1985,  
were carried out on the DNA sequence shown in Table 1  
30 (which has E. coli preference codons), using the oligonucleotides shown in Table 2.

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[illegible]

<u>1L-2 ANALOGS</u>	<u>SEQUENCES (5' → 3')</u>								<u>Length</u>
Leu <sup>80</sup> → Cys <sup>80</sup>	ACG	TGG	ACG	GCA	ATG	AAA	GTT	CTT	24
Lys <sup>8</sup> → Cys <sup>8</sup>	GCT	CTT	CTA	CTT	GTA	AAA	CCC	AGC	24
Pro <sup>47</sup> → Gly <sup>47</sup>	AAA	TTC	TAC	ATG	GGG	AAG	AAA	GCA AC	26
Glu <sup>106</sup> → Lys <sup>106</sup>	GTC	TGC	ATA	TTT	GCA	CAT	GAA	GG	23
Asp <sup>109</sup> → Lys <sup>109</sup>	AGC	GGT	CTC	TTT	TGC	ATA	TTC	GC	23
Ala <sup>112</sup> → Lys <sup>112</sup>	CCA	CGA	TGG	TTT	TGG	TCT	CGT	CT	23
Lys <sup>48</sup> → Glu <sup>48</sup>	CTA	CAT	GCC	GGA	GAA	AGC	AAC	C	22
Thr <sup>51</sup> → Asp <sup>51</sup>	CGA	AGA	AAG	CAG	ACG	AAC	TGA	AAC	24
Lys <sup>54</sup> → Glu <sup>54</sup>	ACC	GAA	CTG	GAA	CAC	CTG	CAG		21
→ Cys <sup>134</sup>	GGA	TCC	TAT	TAG	CAG	GTC	AGA	GTG	24

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Oligonucleotide site-directed mutagenesis was performed by cloning the IL-2 region from XbaI to BamHI, from expression vector pCFM 536 IL-2 into both M13mpl0 and M13mpl1, and the single-stranded phage DNA was  
5 isolated as for DNA sequencing. Although pCFM536 (see U.S. Patent 4,710,473 hereby incorporated by reference) was used, any suitable expression vector could have been used. This DNA was mixed with the synthetic deoxynucleotides of Table 2. The DNA in these mixtures  
10 was allowed to anneal by heating to 65°C and then slowly cooling to room temperature. The oligomers contained the appropriate base changes from the natural recombinant IL-2 sequence in the middle of their sequences. To the annealed DNAs were added ATP, dATP,  
15 dCTP, dGTP, TTP, T4 DNA ligase, and the Klenow fragment of E. coli DNA polymerase I. This reaction allowed the single-stranded primed phage DNAs to convert into covalently closed, double-stranded, circular DNAs. This DNA was transfected directly into E. coli strain JM103  
20 without first purifying the in vitro synthesized double stranded DNA on alkaline sucrose gradients. Many of the plaques from the transfection contained phage DNA with the original recombinant IL-2 sequence, but some contained the IL-2 sequence with the desired base  
25 changes. These plaques were identified by lifting plaques onto nitrocellulose filters, and then hybridizing the filters with the synthetic deoxynucleotide end-labeled with ATP ( $\gamma$ -<sup>32</sup>P). After hybridizing, the filters were washed at a temperature  
30 0-3°C below the melting temperature of the synthetic deoxynucleotide and its complementary DNA sequence. These wash conditions selectively left strong autoradiography signals corresponding to plaques with phage containing the mutated sequence. Positive clones  
35 for each analog were confirmed by DNA sequencing, and these were cloned back into pCFM 536 from XbaI to BamHI.

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Cultures of recombinant IL-2 analogs were grown in media containing 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter at 30°C with shaking until they reached an  $A_{600}$  of 0.5 at which point they  
5 were rapidly heated to 42°C. The flasks were allowed to continue shaking at 42°C for three hours. Cells were harvested by centrifugation at 10,000 x G for 20 minutes at 4°C. Cell pellets were resuspended at 0.4 g wet weight/ml with 1 mM dithiothreitol (DTT) and were passed  
10 twice through a French Pressure Cell at 10,000 psi for 15 minutes at 4°C, and the broken cell supernatants were discarded. The pellets were resuspended in 50 mM Tris, 5 mM EDTA, 5 mM DTT, 0.5 M NaCl, 1% sodium deoxycholate (DOC), pH 9.0 at 0.25 g wet weight original pellet/ml  
15 and were allowed to mix for 30 minutes at room temperature. These mixtures were centrifuged at 10,000 x G for 15 minutes at 14°C and the supernatants were discarded. The pellets were resuspended in H<sub>2</sub>O at 0.15 g wet weight original pellet/ml and centrifuged at  
20 10,000 x G for 15 minutes at 4°C. The supernatants were discarded and the pellets were solubilized at room temperature in 4% sodium laurate, 50 mM Tris, 5% ethanol, 50mM DTT, pH 8.7 at approximately 20-30 mg protein/ml. The solubilized protein was chromatographed  
25 on Sephadex G-75 in 2% sodium laurate, 50 mM Tris, 5% ethanol, pH 8.7. Fractions were analyzed by SDS-PAGE and IL-2 containing fractions of greater than 95% purity were pooled.

Under certain circumstances it was desirable  
30 to have the IL-2 analogs essentially free of pyrogenic substances and endotoxins. This was accomplished by further purification of the molecule. The protein was oxidized in the presence of  $Cu^{2+}$ , concentrated and chromatographed on Sephadex G-75 equilibrated with 1%  
35 laurate/25 mM Tris/ 5% ethanol, pH 8.7. Those fractions containing monomeric forms of IL-2 were pooled and the

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protein was precipitated by addition of an equal volume of ethanol. The pellet was collected by centrifugation, washed with 50% ethanol, and then solubilized in 50% acetic acid. The solution was diluted 50 fold to allow for refolding of the IL-2 analog, concentrated, then diafiltered against a sodium acetate buffer such that the final concentration and pH were 10 mM sodium acetate, pH 4.

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## EXAMPLE 3

## Activity of the IL-2 Analogs

This example relates to the activity of analogs generated in Example 2.

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A number of IL-2 analogs were generated that differ from the native sequence of IL-2 by two amino acids. All analogs tested have Ala at position 125. (Ala<sup>125</sup>)IL-2 (which differs from the native sequence of IL-2 by one amino acid) was used as a positive control for these experiments. (Asp<sup>51</sup>)IL-2 and (Glu<sup>48</sup>)IL-2 are molecules with amino acid changes in the putative receptor-binding B' domain of IL-2. Specifically, in (Asp<sup>51</sup>)IL-2 the neutral threonine at position 51 was replaced by a negatively charged aspartic acid, and in (Glu<sup>48</sup>)IL-2 the positively charged lysine was replaced by the negatively charged glutamic acid. In (Gly<sup>47</sup>)IL-2, the proline between the B and B' domains was replaced with a glycine. This change was predicted to have significant structural consequences for the molecule that would result in a substantial loss of biological activity. These analogs were tested in several in vitro assays for IL-2 activity - the incorporation of <sup>3</sup>H-thymidine into the murine T cell line CTLL-1 or into human peripheral blood leukocytes (hPBL), the generation of lymphokine activated killer cells (LAK cells) from hPBL cultures and for the ability

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to induce IFN-gamma, IL-1 and TNF production in hPBL cultures. These experiments were executed with partially purified material. It is noted that in separate CTL-1 assays using highly purified materials, the specific activity of (Ala<sup>125</sup>)IL-2 was found to be  $7.8 \times 10^6$  U/mg, that for (Glu<sup>48</sup>)IL-2 was found to be  $7.9 \times 10^6$  U/mg, and that for (Asp<sup>51</sup>) IL-2 was found to be  $6.6 \times 10^6$  U/mg. The data from several experiments is shown in Table 3 below.

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TABLE 3  
ASSAY

Exp.	Analog	CIL-1 (Units/mg)	<sup>3</sup> Hdtr into hPBL (cpm)	LAK cells (lytic unit/culture)	IFN-gamma (Units/ml)	IL-1beta (pg/ml)	INF (pg/ml)
1	Ala(125)	1.2x10 <sup>6</sup>	25,987	-	440	-	-
	- Asp(51)	2.0x10 <sup>6</sup>	37,546	-	160	-	-
	- Glu(48)	1.4x10 <sup>6</sup>	25,983	-	100	-	-
	Gly(47)	3x10 <sup>4</sup>	5,788	-	25	-	-
2	Ala(125)	-	-	42x10 <sup>3</sup>	-	-	-
	Asp(51)	-	-	47x10 <sup>3</sup>	-	-	-
	Glu(48)	-	-	49x10 <sup>3</sup>	-	-	-
	Gly(47)	-	-	7.4x10 <sup>3</sup>	-	-	-
3	Ala(125)	1.2x10 <sup>5</sup>	-	97x10 <sup>3</sup>	94	527	4,640
	- Asp(51)	1.2x10 <sup>5</sup>	-	128x10 <sup>3</sup>	55	610	5,600
	- Glu(48)	1.4x10 <sup>5</sup>	-	81x10 <sup>3</sup>	48	290	2,080
	Gly(47)	7.4x10 <sup>3</sup>	-	19x10 <sup>3</sup>	16	130	720
4	Ala(125)	-	-	266x10 <sup>3</sup>	11,400	-	-
	Asp(51)	-	-	266x10 <sup>3</sup>	10,000	-	-
	Glu(48)	-	-	375x10 <sup>3</sup>	6,650	-	-
	Gly(47)	-	-	35x10 <sup>3</sup>	220	-	-

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Taken together, these results indicate that (Asp<sup>51</sup>)IL-2 and (Glu<sup>48</sup>)IL-2 were equivalent to (Ala<sup>125</sup>)IL-2 as T cell mitogens and as inducers of LAK cells. However, (Glu<sup>48</sup>)IL-2 consistently induced less IFN-gamma production from hPBL cultures than did an equal concentration of (Ala<sup>125</sup>)IL-2. In two of three experiments, (Asp<sup>51</sup>)IL-2 was less effective than (Ala<sup>125</sup>)IL-2 in IFN-gamma induction. In one experiment, the production of IL-1 and TNF by the analogs was measured. The induction of these lymphokines by (Asp<sup>51</sup>)IL-2 was equal to that of (Ala<sup>125</sup>)IL-2 while induction by (Glu<sup>48</sup>)IL-2 was approximately 50% as effective. Depending on the assay, (Gly<sup>47</sup>)IL-2 had only 2% to 20% of the biological activity of (Ala<sup>125</sup>)IL-2 as predicted.

## EXAMPLE 4

Alterations in the F-Helix to Include Residues  
with High Preference for Alpha-Helical Structure

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The analogs containing altered F-helix sequence were constructed by a two step procedure. The first step involved the introduction of an Eco R1 restriction site at a region in the gene corresponding to the Glu<sup>116</sup> Phe<sup>117</sup> sequence. This was accomplished by site directed mutagenesis using the primer shown below which codes for the desired change in DNA sequence while leaving the encoded amino acid sequence intact.

30 Mutagenesis Primer for the Introduction of Eco R1 Site

Glu<sup>116</sup>      Phe<sup>117</sup>

5' - CGTG    GAA            TTC            CTGAATCGTT - 3'

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Eco R1

The presence of the Eco RI site allowed for the excision of the portion of the IL-2 gene coding for the F-helix by digestion with Eco RI and Bam HI. The second step involved the replacement of the excised portion of the gene with the synthetic DNA sequence coding for the altered F-helix amino acid sequences. Using this method, the following IL-2 analogs C4 and C5 were constructed:

## 10 Natural Sequence

116                      120                      125                      130  
GAA TTT CTG AAT CGT TGG ATC ACT TTC TGT CAG TCC ATC ATC AGC ACT CTG ACC  
Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr

15 Analogue C4 (Gln<sup>119</sup>Lys<sup>120</sup>Ala<sup>123</sup>Ala<sup>127</sup>Leu<sup>129</sup>Ala<sup>131</sup>)IL-2

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GAA TTC CTG CAG AAA TGG ATC GCT TTC GCA CAG GCT ATC CTG AGC GCA CTG ACC  
Glu Phe Leu Gln Lys Trp Ile Ala Phe Ala Gln Ala Ile Leu Ser Ala Leu Thr

20 Analogs C5 (Gln<sup>119</sup>Lys<sup>120</sup>Phe<sup>121</sup>Ala<sup>123</sup>Gln<sup>127</sup>Leu<sup>129</sup>Gln<sup>130</sup>Ala<sup>131</sup>Ala<sup>133</sup>) IL-2

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GAA TTC CTG CAG AAA TTC ATC GCT TTC GCA CAG CAG ATC CTG CAG GCA CTG GCT  
Glu Phe Leu Gln Lys Phe Ile Ala Phe Ala Gln Gln Ile Leu Gln Ala Leu Ala

Natural Sequence Preferred Structure (A= $\alpha$  helix, B= $\beta$ -sheet, T=turn)

25    Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr  
          T                    T    T    T    B    B    B    B    B    B    B    B    B    B

### Analog C4 Structure

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30   Glu Phe Leu Gln Lys Trp Ile Ala Phe Ala Gln Ala Ile Leu Ser Ala Leu Thr  
      A   A   A   A   A   A   A   A   A   A   A   A   A   A   A   A   A   A

### Analog C5 Structure

[illegible]

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While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended  
5 claims cover all such equivalent variations which come within the scope of the invention as claimed.

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## WHAT IS CLAIMED IS:

1. A polypeptide product of the expression in a host cell of a manufactured gene, said polypeptide  
5 having an amino acid sequence represented by formula [I] below wherein at least one of the 47th, 51st, 80th, 81st, 106th, 109th, 112th, 119th, 120th, 123rd, 127th, 129th, 131st and 133rd original amino acid residue is replaced by a substitution amino acid residue, or  
10 wherein at least two of the 8th, 47th, 48th, 51st, 54th, 80th, 81st, 106th, 109th, 112th, 119th, 120th, 121st, 123rd, 127th, 129th, 130th, 131st, 132nd and 133rd original amino acid residues are replaced by substitution amino acid residues, and wherein X is  
15 selected from the group consisting of Cys, Ala, and Ser:

Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr  
Gln Leu Gln Leu Glu His Leu Leu Leu Asp  
Leu Gln Met Ile Leu Asn Gly Ile Asn Asn  
20 Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu  
Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala  
Thr Glu Leu Lys His Leu Gln Cys Leu Glu [I]  
Glu Glu Leu Lys Pro Leu Glu Glu Val Leu  
Asn Leu Ala Gln Ser Lys Asn Phe His Leu  
25 Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn  
Val Ile Val Leu Glu Leu Lys Gly Ser Glu  
Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu  
Thr Ala Thr Ile Val Glu Phe Leu Asn Arg  
Trp Ile Thr Phe X Gln Ser Ile Ile Ser  
30 Thr Leu Thr.

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2. A polypeptide according to Claim 1 characterized by the presence of one or more of the following substitutions in amino acid sequence:

5                   Lys<sup>8</sup>→Cys<sup>8</sup>;  
                  Pro<sup>47</sup>→Gly<sup>47</sup>;  
                  Thr<sup>51</sup>→Asp<sup>51</sup>;  
                  Leu<sup>80</sup>→Cys<sup>80</sup>;  
                  Arg<sup>81</sup>→Cys<sup>81</sup>;  
10                   Glu<sup>106</sup>→Lys<sup>106</sup>;  
                  Asp<sup>109</sup>→Lys<sup>109</sup>;  
                  Ala<sup>112</sup>→Lys<sup>112</sup>;  
                  Asn<sup>119</sup>→Gln<sup>119</sup>;  
                  Arg<sup>120</sup>→Lys<sup>120</sup>;  
15                   Thr<sup>123</sup>→Ala<sup>123</sup>;  
                  Ser<sup>127</sup>→Ala<sup>127</sup> or Gln<sup>127</sup>;  
                  Ile<sup>129</sup>→Leu<sup>129</sup>;  
                  Ser<sup>130</sup>→Gln<sup>130</sup>;  
                  Thr<sup>131</sup>→Ala<sup>131</sup>;  
20                   Leu<sup>132</sup>→Cys<sup>132</sup>;  
                  Thr<sup>133</sup>→Ala<sup>133</sup>;  
                  addition of Cys<sup>134</sup>;

and optionally one or more of the following substitutions:

25                   Lys<sup>48</sup>→Gly<sup>48</sup>  
                  Lys<sup>54</sup>→Glu<sup>54</sup>  
                  Trp<sup>121</sup>→Phe<sup>121</sup>.

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3. A polypeptide according to Claim 2  
selected from the group consisting of:

5 (Cys<sup>8</sup>) IL-2;  
(Gly<sup>47</sup>) IL-2;  
(Asp<sup>51</sup>) IL-2;  
(Cys<sup>80</sup>) IL-2;  
(Cys<sup>81</sup>) IL-2;  
(Lys<sup>106</sup>) IL-2;  
10 (Lys<sup>109</sup>) IL-2;  
(Lys<sup>112</sup>) IL-2;  
(Cys<sup>132</sup>) IL-2;  
(Cys<sup>134</sup>) IL-2;

15 4. A polypeptide according to Claim 1 wherein  
said polypeptide also has one or more of the following  
alterations in the amino acid sequence:

- (a) deletion and/or replacement of amino acid  
residues providing sites of intramolecular folding;  
20 (b) deletion of terminal amino acid residues;  
(c) addition of amino acid residues to  
terminal amino acid residues;  
(d) deletion and/or replacement of amino acid  
residues providing sites of hydrolytic instability under  
25 highly acidic conditions;  
(e) replacement of amino acid residues with  
glutamine residues;  
(f) replacement of amino acid residues with  
phenylalanine residues;  
30 (g) deletion and/or replacement of tryptophan  
residues;  
(h) deletion and/or replacement of asparagine  
residues;  
(i) deletion and/or replacement of cysteine  
35 residues;  
(j) replacement of amino acid residues with  
serine residues; and

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(k) replacement of amino acid residues with alanine residues.

5           5. A polypeptide product according to Claim 1  
in which at least one of: the 8th, 80th and 132nd amino  
acid residues is replaced by a substitution amino acid  
residue which permits attachment of a ligand, and/or a  
134th amino acid residue which permits attachment of a  
ligand, is added.

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6. A polypeptide as in Claim 5 wherein said  
substitution amino acid residue and said 134th amino  
acid residue are Cys.

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7. A polypeptide as in Claim 5 coupled via  
said 8th, 80th, 81st, 132nd or 134th amino acid residue,  
to a label.

8. A polypeptide as in Claim 5 coupled via  
20 said 8th, 80th, 81st, 132nd or 134th amino acid residue,  
to an enzymatic moiety.

9. A polypeptide as in Claim 5 coupled via  
said 8th, 80th, 81st, 132nd or 134th amino acid residue,  
25 to a toxin.

10. A polypeptide as in Claim 5 coupled via  
said 8th, 80th, 81st, 132nd or 134th amino acid residue,  
to a drug.

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11. A polypeptide as in Claim 5 coupled via  
said 8th, 80th, 81st, 132nd or 134th amino acid residue,  
to an antibody or mitogen.

35           12. An antibody specifically binding the  
polypeptide product of Claim 1 but which does not cross  
react with naturally occurring human IL-2.



13. An antibody as in Claim 12 which is tagged.

14. A manufactured gene capable of directing the synthesis in a selected host cell of the polypeptide of Claim 1.

15. A manufactured gene capable of directing the synthesis in a selected host cell of the polypeptide of Claim 2.

16. A manufactured gene according to Claim 14 wherein the base sequence includes one or more codons, selected from among alternative codons specifying the same amino acid, on the basis of preferential expression characteristics of the codon in a projected host cell.

17. A manufactured gene according to Claim 14 wherein the base sequence includes one or more codons, selected from among alternative codons specifying the same amino acid, on the basis of preferential expression characteristics of the codon in E. coli.

18. A manufactured gene according to Claim 14 wherein base codons specifying the polypeptide include initial and/or terminal codons respectively specifying additional initial and/or terminal amino acids in the polypeptide synthesized.

19. A manufactured gene according to Claim 18 wherein said initial codons specifying additional initial amino acids are codons specifying an initial methionine residue.

20. A manufactured gene according to Claim 14 wherein the base codons specifying the polypeptide are preceded and/or followed by and/or include a sequence of bases comprising a portion of a base sequence which provides a recognition site for restriction endonuclease enzyme cleavage.

21. A manufactured gene according to Claim 14 wherein the base codons specifying the polypeptide are preceded by a sequence of bases comprising a portion of a base sequence which provides a site for ribosome binding.

22. A manufactured gene according to Claim 21 wherein said ribosome binding site is specified by the sequence 5'-CAA GGA GGT-3'.

23. A manufactured gene according to Claim 14 which is labelled.

24. A biologically functional DNA transformation vector including the manufactured gene of Claims 14 or 15.

25. A transformed cell with a vector including a manufactured gene of Claims 14 or 15.

26. A method of removing pyrogens from an IL-2 solution containing pyrogens comprising the steps of:

(a) adjusting the pH of a detergent free IL-2 solution to a pH so that the pyrogens form aggregates of molecular weight greater than the molecular weight of monomeric IL-2; and

(b) separating the aggregates from the IL-2 solution by size.

27. A method as in Claim 26 wherein step (b) comprises repeatedly i) diluting the IL-2 solution and ii) separating the aggregates from the IL-2 solution.

5           28. A method as in Claim 26 wherein, step (b) comprises separating the aggregates from the IL-2 solution by ultrafiltration or size exclusion chromatography.

10           29. An IL-2 analog which is the product of the expression in a host cell of a manufactured gene, said analog having one or more of the biological properties of naturally occurring IL-2, wherein at least two original amino acids in helix A and/or helix F have  
15           been replaced by substitution amino acids, said substitution amino acids altering the amphiphilicity of each helix containing a substitution amino acid.

            30. An IL-2 analog as in Claim 29 wherein the  
20           substitution amino acids change the ratio in each helix containing a substitution amino acid, of amino acids having hydrophobic side chains to amino acids having hydrophilic side chains.

25           31. An IL-2 analog as in Claim 29 wherein said at least two original amino acids are amino acids having hydrophobic side chains and said substitution amino acids are amino acids having hydrophilic side chains.

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            32. An IL-2 analog as in Claim 29 wherein each of said substitution amino acids has a greater preference for an alpha-helical structure than the original amino acid it replaces.

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33. An IL-2 analog which is the product of the expression in a host cell of a manufactured gene, said analog having one or more of the biological properties of naturally occurring IL-2, wherein at least one original amino acid in helix E, and/or at least two original amino acids in helix A, B, and/or B' have been replaced by substitution amino acids, each of said substitution amino acids having a different charge than the original amino acid it replaces.

10

34. An IL-2 analog as in Claim 33 wherein said original amino acids are hydrophilic amino acids.

35. An IL-2 analog as in Claim 33 wherein said original amino acids are charged amino acids.

36. IL-2 analog as in Claim 33 wherein each of said substitution amino acids has a greater preference for an alpha-helical structure than the original amino acid it replaces.

37. An IL-2 analog which is the product of the expression in a host cell of a manufactured gene, said analog having one or more of the biological properties of naturally occurring IL-2, wherein at least one original amino acid in helix C, D and/or E, and/or at least two original amino acids in helix A, B, B', and/or F have been replaced by substitution amino acids, each of said substitution amino acids having a greater preference for an alpha-helical structure than the original amino acid it replaces.

38. An IL-2 analog as in Claim 37 wherein said original amino acids in helix A, B, B', E and/or F have been replaced by said substitution amino acids, each of said substitution amino acids having a greater

- 43 -

preference for an alpha-helical structure than the original amino acid it replaces.

39. An IL-2 analog as in Claim 37 wherein  
5 said at least one amino acid in helix F has been replaced by said replacement amino acid which has a greater preference for an alpha-helical structure.

40. An IL-2 analog as in Claim 37 wherein  
10 said substitution amino acids are selected from the group consisting of: Glu, Met, Ala, Leu, Lys, Phe, Gln, Trp, Ile, Val, Asp, and His.

41. An IL-2 analog which is the product of  
15 the expression in a host cell of a manufactured gene, said analog having one or more of the biological properties of naturally occurring IL-2, wherein at least one original amino acid in helix A, B', C, D, and/or E, and/or at least two original amino acids in helix B  
20 and/or F have been replaced by substitution amino acids, each of said substitution amino acids having a greater preference for an alpha-helical structure than the original amino acid it replaces, and each of said substitution amino acids altering the amphiphilicity of  
25 each helix containing a substitution amino acid.

42. An IL-2 analog as in Claim 41 wherein if the original amino acid is hydrophilic, the substitution amino acid is selected from the group consisting of:  
30 Met, Leu, Phe, Trp, Ile, and Val; and if the original amino acid is hydrophobic the substitution amino acid is selected from the group consisting of: Glu, Ala, Lys, Gln, Asp, and His.

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43. A peptide which is the product of the expression in a host cell of a manufactured gene, said peptide being capable of binding the IL-2 receptor and including at least one of helices A, B, B', and E, and  
5 not including helices C, D, and F.

44. A peptide as in Claim 43 wherein at least one original amino acid in helices A, B, B' and E is replaced by a substitution amino acid which has greater  
10 preference for an alpha-helical structure than the original amino acid it replaces.

45. A pharmaceutical composition comprising an effective amount of a polypeptide according to  
15 Claim 1 and a pharmaceutically acceptable diluent, adjuvant or carrier.

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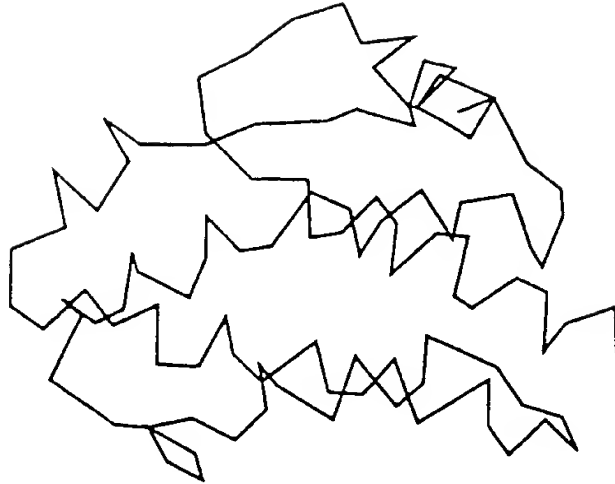
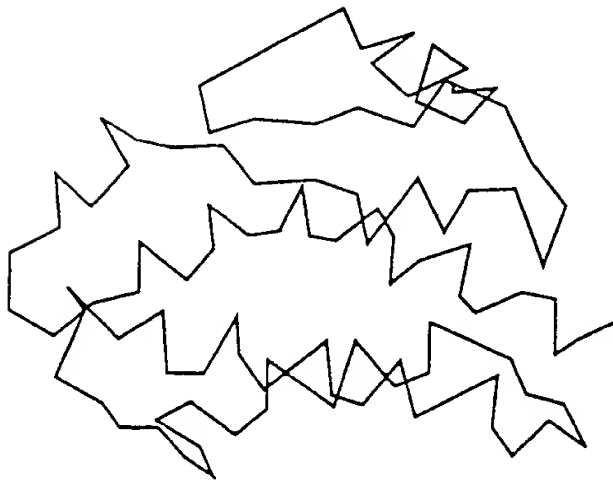


FIG 1-A



**SUBSTITUTE HEET**

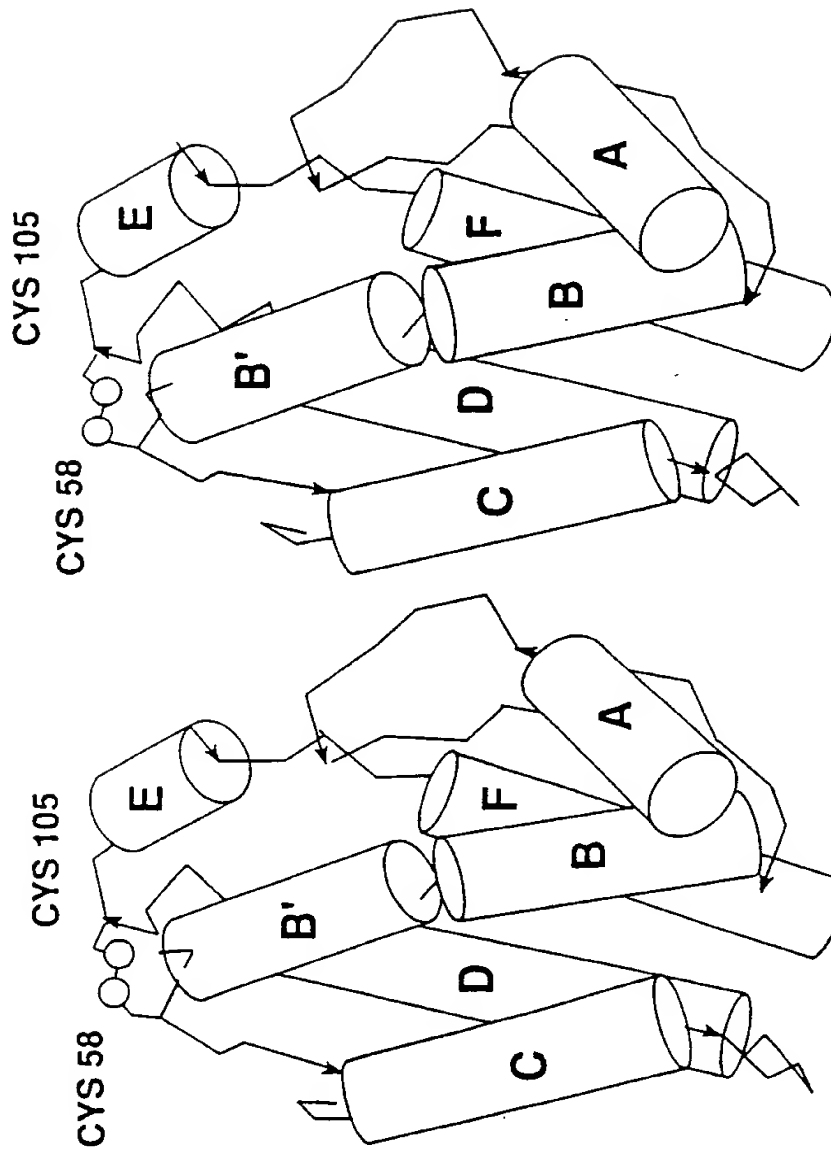
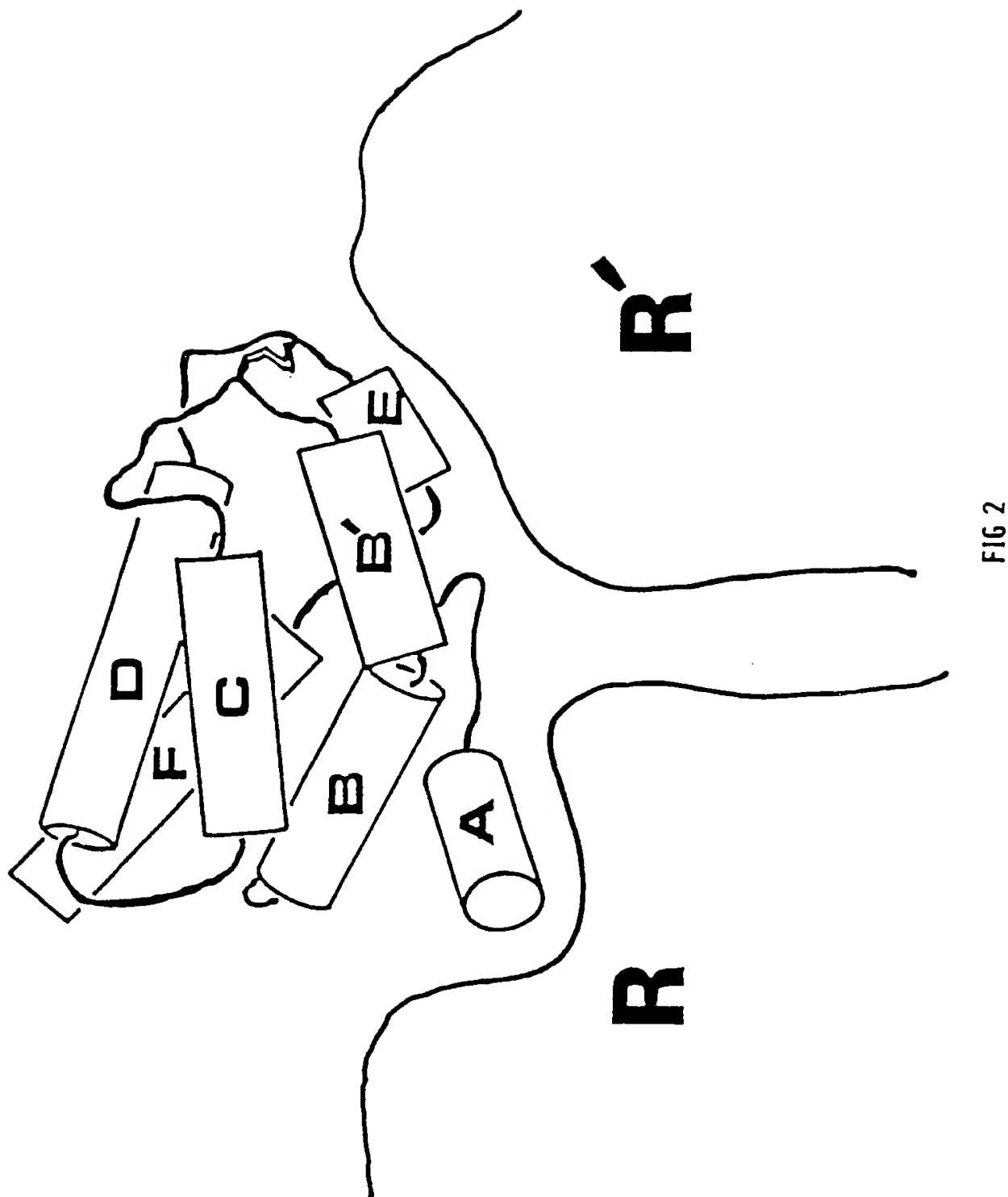


FIG 1-B

SUBSTITUTE SHEET





SUBSTITUTE SHEET



**SUBSTITUTE SHEET**

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02917

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC INT. CL. 4 C07K 13/00, 17/00, 3/26; A61K 45/00; C12N 15/00; C07H 17/00, 21/04 US CL. 530/351, 387, 402, 412, 417; 435/68, 172.3, 320; 536/27; 424/85.2;																							
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched <sup>7</sup></div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%;">Classification System</th> <th style="width: 75%;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">U.S.</td> <td style="padding: 5px;">530/351, 387, 402, 404, 405, 406, 409, 412, 414, 417; 435/68, 70, 172.1 172.3, 320; 536/27; 424/85.2, 85.1, 85.91, 1.1; 514/2, 8; 935/10, 11, 22</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	U.S.	530/351, 387, 402, 404, 405, 406, 409, 412, 414, 417; 435/68, 70, 172.1 172.3, 320; 536/27; 424/85.2, 85.1, 85.91, 1.1; 514/2, 8; 935/10, 11, 22																	
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Computer search on CAS, Dialog and PIR. For: IL-2 muteins, analogs, derivatives; DNA, antibodies, purification; and sequence search.																							
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category <sup>*</sup></th> <th style="width: 60%;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 30%;">Relevant to Claim No. <sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X Y</td> <td style="vertical-align: top;">Journal of Biological Chemistry, Vol. 262, Issued 25 April 1987, :Structure-Function Analysis of Human Interleukin-2," (Ju), pages 5723-31, See all.</td> <td style="vertical-align: top;">1-6, 12-25, 29-45 <hr/>7-11</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">Science, Vol. 234, Issued 17 October 1986, "Structure Activity Studies of Interleukin-2;" (Cohen), pages 349-351, See page 349, 351.</td> <td style="vertical-align: top;">1-25, 29-45</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">Science, Vol. 238, Issued 13 December 1987, "Three Dimensional Structure of Interleukin-2," (Brandhuber), pages 1707-09, See all.</td> <td style="vertical-align: top;">1-45</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X Y</td> <td style="vertical-align: top;">PCT, A, 85/00817, (Souza), 28 February 1985, See pages 3-4, 27.</td> <td style="vertical-align: top;">1-25, 29-45 <hr/>26-28</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X Y</td> <td style="vertical-align: top;">EP, A, 0,200,280, (Koths), 12 October 1986, See pages 6-8, 12 and claims.</td> <td style="vertical-align: top;">1-6, 14-25, 29-45 <hr/>7-13, 26-28</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X Y</td> <td style="vertical-align: top;">PCT, A, 87/04714, (Nishida), 13 August 1987, See abstract.</td> <td style="vertical-align: top;">1-6, 14-25, 29-45 <hr/>7-13, 26-28</td> </tr> </tbody> </table>			Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X Y	Journal of Biological Chemistry, Vol. 262, Issued 25 April 1987, :Structure-Function Analysis of Human Interleukin-2," (Ju), pages 5723-31, See all.	1-6, 12-25, 29-45 <hr/> 7-11	Y	Science, Vol. 234, Issued 17 October 1986, "Structure Activity Studies of Interleukin-2;" (Cohen), pages 349-351, See page 349, 351.	1-25, 29-45	Y	Science, Vol. 238, Issued 13 December 1987, "Three Dimensional Structure of Interleukin-2," (Brandhuber), pages 1707-09, See all.	1-45	X Y	PCT, A, 85/00817, (Souza), 28 February 1985, See pages 3-4, 27.	1-25, 29-45 <hr/> 26-28	X Y	EP, A, 0,200,280, (Koths), 12 October 1986, See pages 6-8, 12 and claims.	1-6, 14-25, 29-45 <hr/> 7-13, 26-28	X Y	PCT, A, 87/04714, (Nishida), 13 August 1987, See abstract.	1-6, 14-25, 29-45 <hr/> 7-13, 26-28
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>																							
<b>IV. CERTIFICATION</b> <table style="width: 100%;"> <tr> <td style="width: 50%; vertical-align: top;">           Date of the Actual Completion of the International Search             20 September 1989             International Searching Authority             ISA/US         </td> <td style="width: 50%; vertical-align: top;">           Date of Mailing of this International Search Report   <div style="font-size: 1.5em; font-weight: bold;">18 OCT 1989</div>             Signature of Authorized Officer              GARNETTE D. DRAPER         </td> </tr> </table>			Date of the Actual Completion of the International Search  20 September 1989  International Searching Authority  ISA/US	Date of Mailing of this International Search Report  <div style="font-size: 1.5em; font-weight: bold;">18 OCT 1989</div>  Signature of Authorized Officer  GARNETTE D. DRAPER																			
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## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P, $\frac{X}{Y}$	US, A, 4,761,375, (Clark), 2 August 1988, See claims	4,30-45, 12-13
Y	US, A, 4,675,382, (Murphy), 23 June 1987, See Cols. 3-4.	9
Y	US, A, 4,636,463, (Altman), 13 January 1987, See cols. 3,5,7 and claims.	1-7,11-13, 29-45
Y	US, A, 4,568,640, (Rubin), 4 February 1986, See claims.	1-6,29-45
Y	EP, A, 0,212,914, (Deeley), 3 April 1987, See abstract and claims.	1-6,14-25, 29-45
Y	Gene, vol. 34, Issued 1985, "Cassette Mutagenesis: An Efficient Method for Generation of Multiple Mutations at Defined Sites," (Wells), pages 315-23, See page 315.	1-6,14-25, 29-45
Y	J. Immunological Methods, Vol. 61, Issued 1983, "Removal of Gram-Negative Endotoxin from Solutions by Affinity Chromatography," (Issekutz), pages 275-81, See page 279.	26-28
T,P	Nature, Vol. 339, Issued 18 May 1989, "Structural Plasticity Broadens the Specificity of an Engineered Protease," (Bone), pages 191-195.	1-25, 29-45
T,P	Chemical & Engineering News, Issued 10 April 1989, "Deciphering the Rules of Protein folding," (King), pages 32-54.	1-25 29-45

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows: Group I, claims 1-6, 29-45 to IL-2 analogs. Group II, claims 7-11 to IL-2 analog conjugates. Group III, claims, 12-13 to IL-2 analog antibodies. Group IV, claims 14-25 to genes and vectors. Group V, claims 26-28 to a method of removing pyrogen from IL-2.  
(See Attachments)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
  
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
  
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

